CHAPTER IV
RESULTS AND DISCUSSIONS

4.1 Characterization of Mineral Medium, Palm Oil and Glucose

One of the most important factors for biosurfactant production was culture medium. The compositions of culture medium in this study composed of palm oil and glucose that were used as carbon sources and MM was used as nutrient source for the biosurfactant production by *Pseudomonas aeruginosa* SP4. MM contained $NaNO_3$, K_2HPO_4, and KH_2PO_4, which an amount of each chemical was varied for each oil-to-glucose ratios to keep C/N and C/P constant, $MgSO_4 \cdot 7H_2O$ (0.5 g/l), KCl (0.1 g/l), and $FeSO_4 \cdot 7H_2O$ (0.01 g/l). The C/N and C/P ratios of culture medium were kept constantly at 16/1 and 14/1, respectively, because they were reported to be the optimum ratios for the rhamnolipid production by *Pseudomonas aeruginosa* (Pornsunthorntawee *et al.*, 2009). To calculate the C/N and C/P ratios, the results obtained from TOC, TN, and TP measurements were used. Table 4.1 shows that characteristics of palm oil and Table 4.2 shows that characteristics of MM included glucose at different oil-to-glucose ratios that used in this study. It was found that COD and TOC of MM with glucose at all of oil-to-glucose ratios were low while those of palm oil were relatively high. However, pH of MM was higher than that of palm oil. SS of MM and palm oil were zero, and there was no biosurfactant in MM due to a high surface tension of about 71 mN/m, which was close to that of pure water (72 mN/m).

Table 4.1 Characteristics of palm oil and glucose

Parameters		Palm oil
COD	(mg/l)	2,473,459
TOC	(mg/l)	563,489
Total nitrogen	(mg/l)	1,500
Total phosphorus	(mg/l)	90
Suspended solids	(mg/l)	0
Surface tension	(mN/m)	-
pH		4.6
Surfactant concentration	(xCMC)	0

Table 4.2 Characteristics of mineral medium (include glucose) at different oil-to-glucose ratios

Parameters		Mineral medium at different oil-to-glucose ratios					
		10/1	20/1	30/1	40/1	60/1	without glucose
COD	(mg/l)	563.6	281.8	187.9	140.9	93.9	0
TOC	(mg/l)	237.7	118.9	79.2	59.4	39.6	0
Total nitrogen	(mg/l)	242	234	232	231	229	227
Total phosphorus	(mg/l)	287	279	276	275	273	270
Suspended solids	(mg/l)	0	0	0	0	0	0
Surface tension	(mN/m)	71.80	71.57	70.87	71.87	71.47	71.66
pH		7.34	7.29	7.28	7.30	7.31	7.29
Surfactant concentration	(xCMC)	0	0	0	0	0	0

4.2 Effect of Oil-to-Glucose Ratios on Biosurfactant Production

To determine performance of the reactors, 6 parameters were investigated. There are chemical oxygen demand (COD), % oil removal, surface tension (ST), mixed liquor suspended solid (MLSS), suspended solid (SS), and pH. The effect of oil-to-glucose ratios on the reactor performance were studied at six different oil-to-glucose ratios (10/1, 20/1, 30/1, 40/1, 60/1, and without glucose added). An oil loading rate (OLR) and cycle time were 2 kg/m^{3}days and 2 d/cycle time, respectively, because it was reported these conditions were the optimum conditions for biosurfactant production (Pornsunthorntawee *et al.*, 2009).

4.2.1 Effect of Oil-to-Glucose ratio on COD removal

Chemical Oxygen Demand (COD) measurement was the method to determine the concentration of organic compounds in the sample. It expressed the oxygen concentration in terms of mg/l, indicating the mass of oxygen consumed by microorganisms per liter of solution. In this experiment, CODs of both influent and effluent were measured in order to quantify soluble organic carbon (such as biosurfactant and metabolites), and also to determine the degradation capability of the mi-

crobe for utilizing organic substances as their nutrients. Therefore, the constant COD could be used to indicate the steady-state condition. The influent COD mainly came from palm oil and glucose since COD of MM was zero. The effluent COD was related to the remaining palm oil, the excreted biosurfactant, and some metabolites because glucose was used completely during the operation, the glucose concentration in the effluent was quantified by Glucose (HK) Assay Kit (GAHK-20, Sigma Aldrich) and UV-VIS spectrometer (2550, Shimadzu).

As shown in Figure 4.1 and Figure 4.2, the effluents COD and COD removal, respectively, the COD removal of the oil-to-glucose ratio without glucose added was approximately 79-81 % along the operation. At the beginning, the 10/1 oil-to-glucose ratio could provide the highest COD removal because glucose that added to the mineral medium could support the growth of microorganisms in the reactor since it was easy to consume whereas after the steady-state, 8^{th} operation-day, this oil-to-glucose ratio provided the lowest COD removal. Interestingly, after steady state, the oil-to-glucose ratio of 40/1 gave the highest COD removal. The results suggest that an addition of glucose can promote the oil uptake because glucose can be easily consumed by the microbe, leading to higher microbial concentration. As a result, the higher oil in the system can be consumed by the microbe. However, if glucose is added too much, the system will have too high COD in the system, resulting in lower COD removal. Hence, for the studied system, an oil-to-glucose ratio of 40/1 is considered to be the optimum ratio as shown in Table 4.3.

Table 4.3 The average COD of influent, effluent, and COD removal during steady-state operation with different oil-to-glucose ratios

Oil-to-Glucose ratio	Influent COD (mg/l)	Effluent COD (mg/l)	COD removal (%)
Without glucose	33291	6280	81.14
60:1	33385	5739	82.83
40:1	33439	4968	85.14
30:1	33479	5927	82.30
20:1	33573	6251	81.38
10:1	33854	9357	72.36

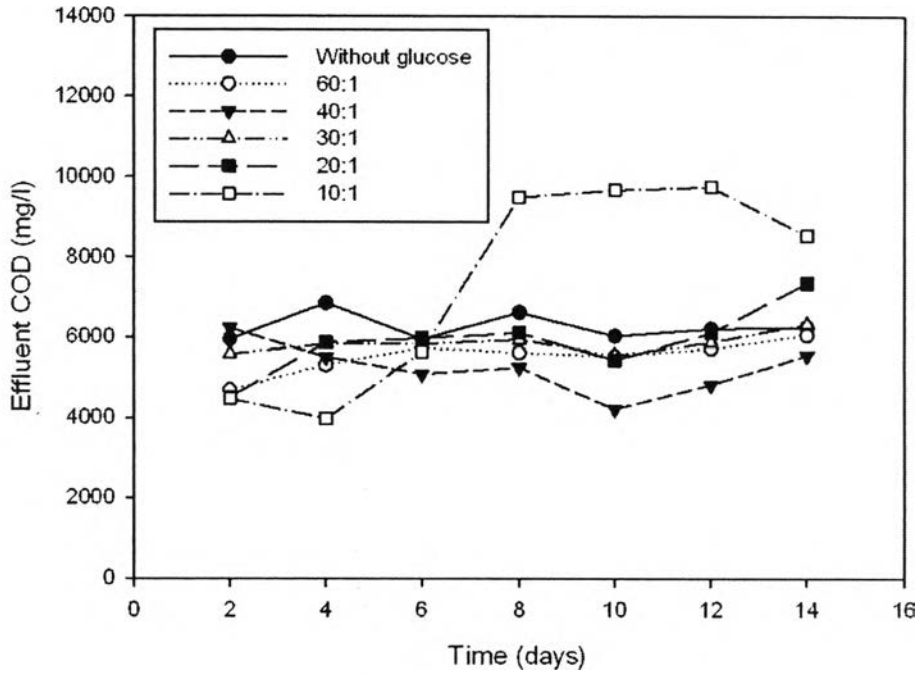

Figure 4.1 Effluent COD as a function of operation time with an oil loading rate of 2 kg/m^{3}d at different oil-to-glucose ratios (days 2-14).

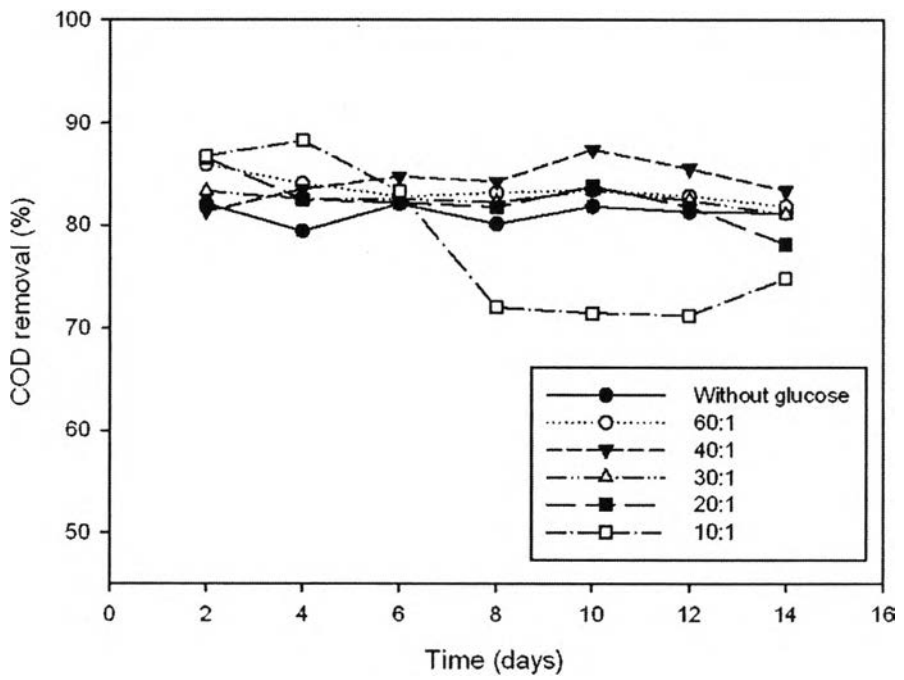

Figure 4.2 COD removal as a function of operation time with an oil loading rate of 2 kg/m^{3}d at different oil-to-glucose ratios (days 2-14).

4.2.2 Effect of Oil-to-Glucose Ratio on Oil Removal

Oil removal was used to indicate the palm oil consumption by the microbe for the biosurfactant production. Figure 4.3 shows that an average the percentage of oil removal during steady-state at different oil-to-glucose ratios. It was found that oil removal percentages of 52.37%, 56.85%, 77.69%, 48.26%, 46.26%, and 41.02% were observed at oil-to-glucose without glucose added, 60/1, 40/1, 30/1, 20/1, and 10/1 oil-to-glucose ratios, respectively. It exhibited that at 60/1, and 40/1 oil-to-glucose ratio, microorganisms could consume palm oil higher than oil-to-glucose ratio without glucose added. On the other hand, microorganisms could not effectively consumed palm oil at 30/1, 20/1, 10/1 oil-to-glucose ratio. This caused by the amount of glucose added in the reactor, when over loading of glucose, microorganisms could consume only glucose then high oil remaining in the reactor. The results also suggested that the microbial ability to degrade palm oil depended on oil-to-glucose ratio.

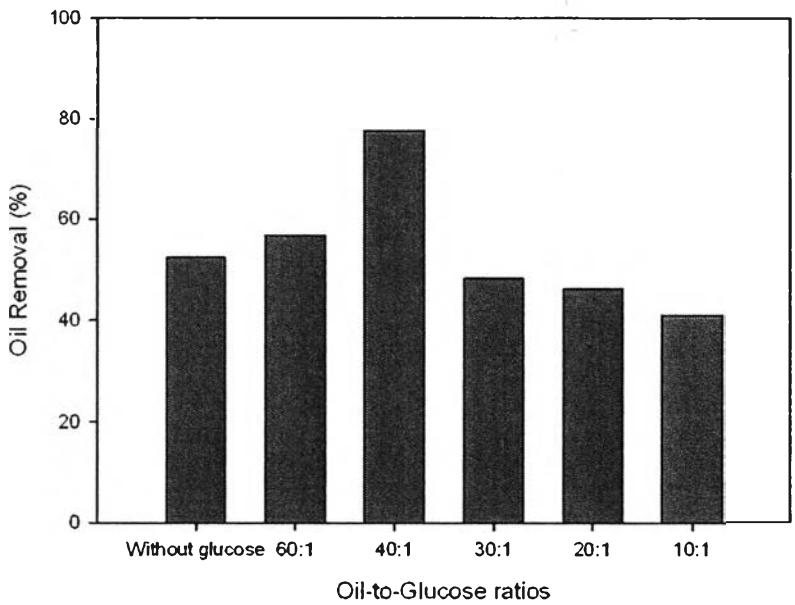

Figure 4.3 Oil removal percentage during steady-state operation with the different oil-to-glucose ratios

4.2.3 Effect of Oil-to-Glucose Ratio on Surface Tension and Surface Tension Reduction

Surface activity of a surfactant was determined by its ability to lower surface tension of medium which was surface free energy per unit area that required bringing a surfactant molecule from the bulk phase to the surface (Rosen, 1978). When the surfactant concentration was higher than the CMC, it was supposed that additional surfactant molecules aggregated into micelles in the bulk phase and did not contribute to significant future change at the interface. Therefore, a surface tension of the medium remains constant at the surfactant concentration which is higher than its CMC. In this study, a surface tension of the culture supernatant of *Pseudomonas aeruginosa* SP4 was measured to estimate the extent of biosurfactant production. The higher surface tension reduction percentage was, the higher biosurfactant yield would be.

Figure 4.4 and Figure 4.5 shows profile of a surface tension, and a surface tension reduction, respectively, of the culture supernatant at different oil-to-glucose ratios as a function of operation time, while Table 4.4 shows an average surface tension and surface tension reduction during the steady state at different oil-to-glucose ratios. It was found that a surface tension of the culture medium at oil-to-glucose ratio without glucose added, 60/1, 40/1, 30/1, 20/1, and 10/1 decreased from about 72 mN/m to 33.03, 30.19, 29.86, 29.93, 30.31, and 29.51 mN/m, providing the surface tension reduction percentage of 53.90%, 57.76%, 58.45%, 57.77%, 57.78% and 58.90%, respectively. It was reported that the biosurfactant production caused the reduction of surface tension of the culture medium. For example, the biosurfactant produced by *Pseudomonas aeruginosa* 47T2 using olive oil and sunflower oil as carbon sources could lower surface tensions of culture media to 34 and 37 mN/m, respectively (Haba *et al.*,2000). The rhamnolipid biosurfactant-containing supernatant obtained from batch culture of *Pseudomonas aeruginosa* J4 reduced a surface tension of pure water from 72 to 31 mN/m (Wei *et al.*,2005).

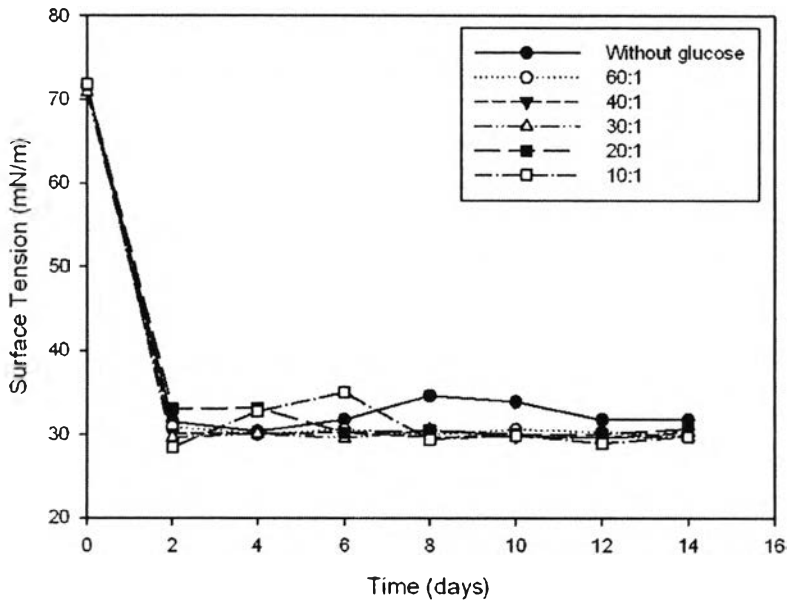

Figure 4.4 Profile of surface tension at different oil-to-glucose ratios as a function of operation time with an oil loading rate of 2 kg/m^{3}d.

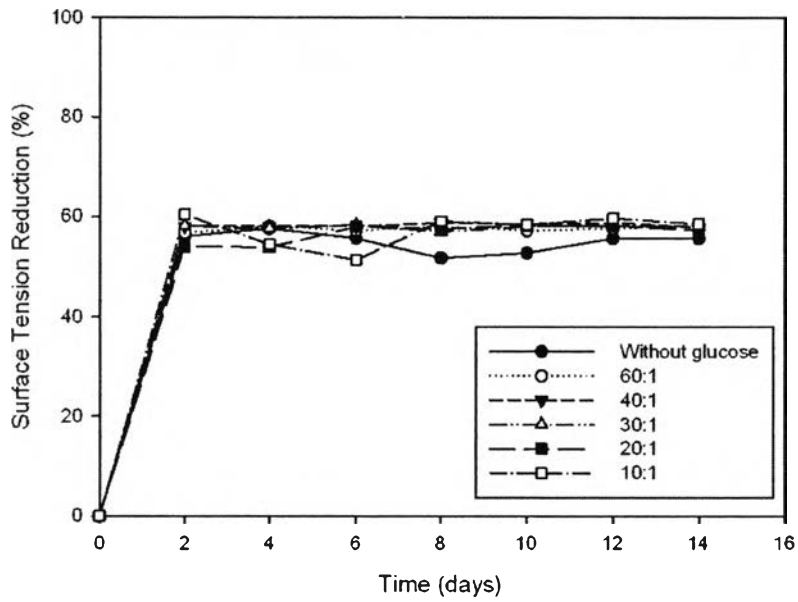

Figure 4.5 Profile of surface tension reduction at different oil-to-glucose ratios as a function of operation time with an oil loading rate of 2 kg/m^{3}d.

Table 4.4 The average surface tension and surface tension reduction during steady-state operation (days 8-14) of oil-to-glucose ratio with different oil-to-glucose ratios

Oil-to-Glucose ratio	Surface Tension (mN/m)	Surface Tension Reduction (%)
Without glucose	33.03	53.90
60:1	30.19	57.76
40:1	29.86	58.45
30:1	29.93	57.77
20:1	30.31	57.78
10:1	29.51	58.90

4.2.4 Microbial Concentration

4.2.4.1 Mixed Liquor Suspended Solid (MLSS)

MLSS represented the concentration of microorganisms in the reactor. The sample was taken during the aeration step in order to determine the concentration of suspended solids (both organic and inorganic substances) in the reactor. The taken sample was filtrated through a glass fiber filter to obtain suspended solid. The filtrated sample is subsequently dried and weighed to determine the amount of mixed liquor suspended solids in terms of mg/l. Figure 4.6 shows an average MLSS during the steady state of oil-to-glucose ratio without glucose added, 60/1, 40/1, 30/1, 20/1, and 10/1 oil-to-glucose ratio were 2915, 3165, 5212.5, 7565, 8402.5, and 10622.5 mg/l, respectively. It was found that an increase in the glucose added could increase the microbial concentration in the reactor. The results suggested that glucose added could enhance the ability of microbial growth during the operation.

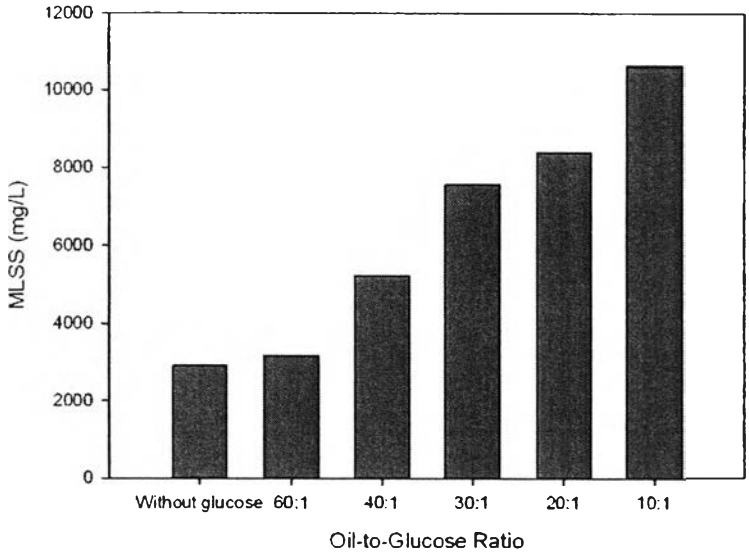

Figure 4.6 An average MLSS during steady state operation (days 8-14) at different oil-to-glucose ratios.

4.2.4.2 Suspended Solid (SS)

SS referred to solid materials including both organic and inorganic substances in the medium. The same methods were used to determine SS as MLSS which are the suspended solid could be separated from the medium by filtrating through a glass fiber filter. The filtrated sample was subsequently dried and weighed to determine the amount of total suspended solids in terms of mg/l. Although the SS method was originally developed to use with wastewater samples, it has been widely adapted to stream samples. The SS method was acceptable for regulatory purposes and it was a relatively-inexpensive laboratory procedure. In this experiment, SS was measured during the settle step and used to represent the cells wash out. Figure 4.7 shows effluent SS at different oil-to-glucose ratio. Effluent SS at oil-to-glucose ratio without added, 60/1, 40/1, 30/1, 20/1, and 10/1 oil-to-glucose ratio was 490, 602.5, 1525, 3620, 4565, and 6207.5 mg/l, respectively. It was observed that 10/1 oil-to-glucose ratio could obtain the highest MLSS and SS, so it was possible that the higher glucose added promoted the microbial growth in the reactor. According to Rashedi, H. et al. reported in 2006 that Production of rhamnolipids by *Pseudomonas aeruginosa* glowing on glucose gave 7.1 g/l of cell dry mass which was higher than ethanol (0.9 g/l), sucrose (0.4 g/l), maltose (0.4 g/l), glycerol (0.4

g/l), and dates (5.2 g/l). Also it was reported that *Pseudomonas aeruginosa* EM1 provided the highest biomass when compared glucose to the other carbon sources (glycerol, sucrose, hexane, oleic acid, olive oil, and soybean oil) (Wu, J.-Y. et al., 2008)

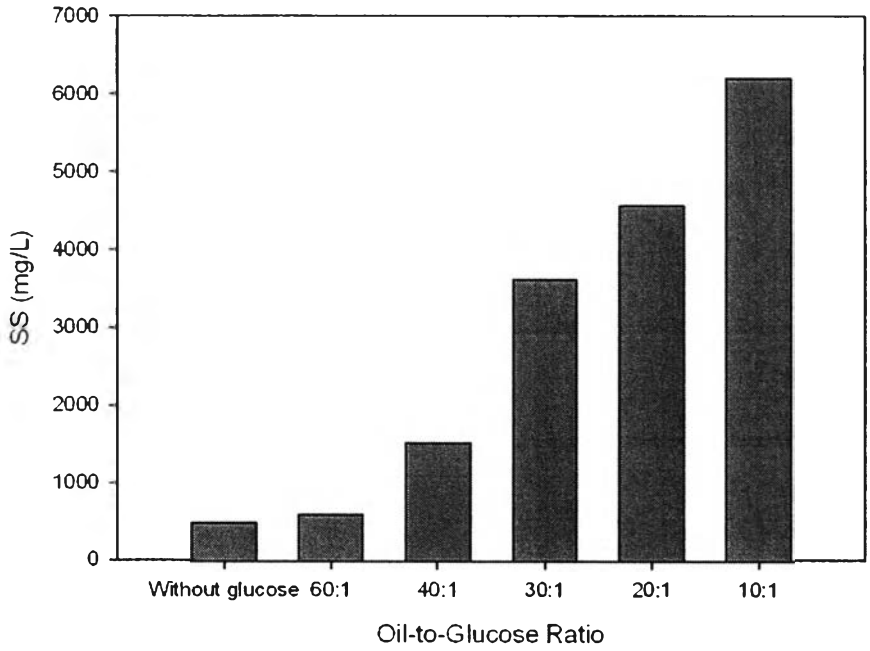

Figure 4.7 An average effluent SS during steady-state operation at (days 8-14) at different oil-to-glucose ratios.

4.2.5 The Effluent pH

pH of the culture medium was a significant environmental factor in the biosurfactant production, because pH could directly affect cellular growth or activity. A little change in pH of the culture medium could significantly alter the biosurfactant productivity. In this study, after removing microbial cells by centrifugation, pH of the supernatant was measured as a function of the oil-to-glucose ratios. Figure 4.8 shows profile of pH at different oil-to-glucose ratios as a function of operation time. Table 4.5 displays the average effluent pHs of oil-to-glucose ratio without glucose added, and all of oil-to-glucose ratios were approximately 7.5-8.2, while 40/1 oil-to-glucose ratio was about 7.9, which was the most suitable pH for the production of

biosurfactant by *Pseudomonas aeruginosa* SP4 using SBRs. It was reported that Rhamnolipid production in *Pseudomonas* spp. was at its maximum at a pH range from 6 to 6.5 and decreased sharply above pH 7 (Desai, J. D., and Banat, I. M., 1997). This might be resulted from the different bacterial strain and the different composition of carbon sources used for the biosurfactant production.

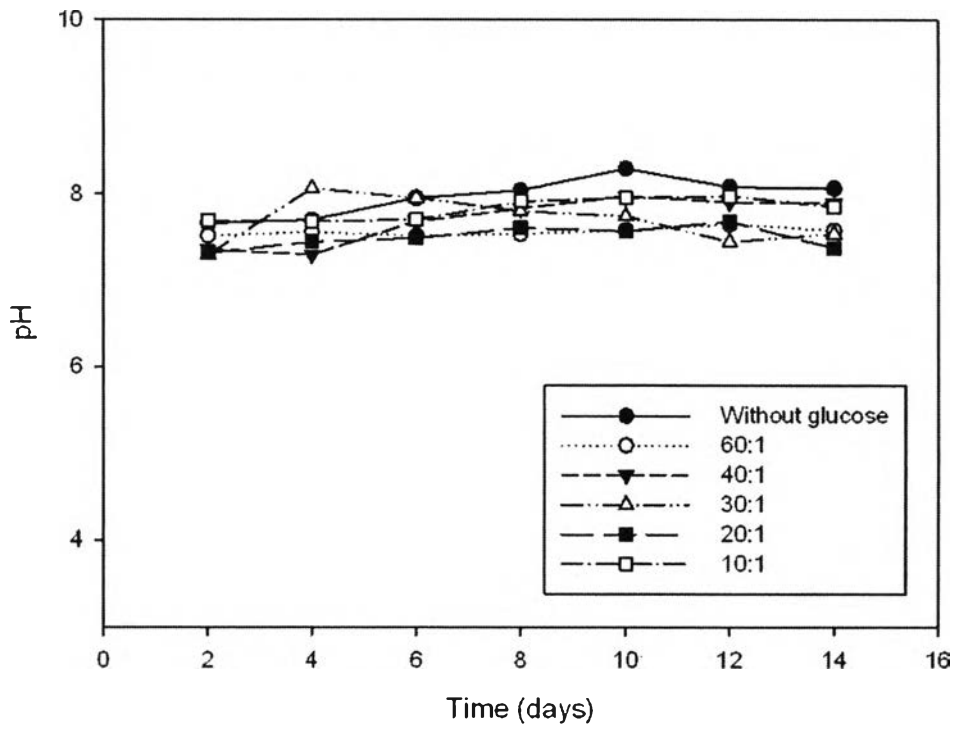

Figure 4.8 Profile of pH at different oil-to-glucose ratios as a function of operation time with an oil loading rate of 2 kg/m^{3}d.

Table 4.5 The average effluent pH during the steady-state operation at (days 8-14) at different oil-to-glucose ratios

Oil-to-Glucose ratio	Effluent pH
Without glucose	8.12
60:1	7.59
40:1	7.90
30:1	7.63
20:1	7.91
10:1	7.92

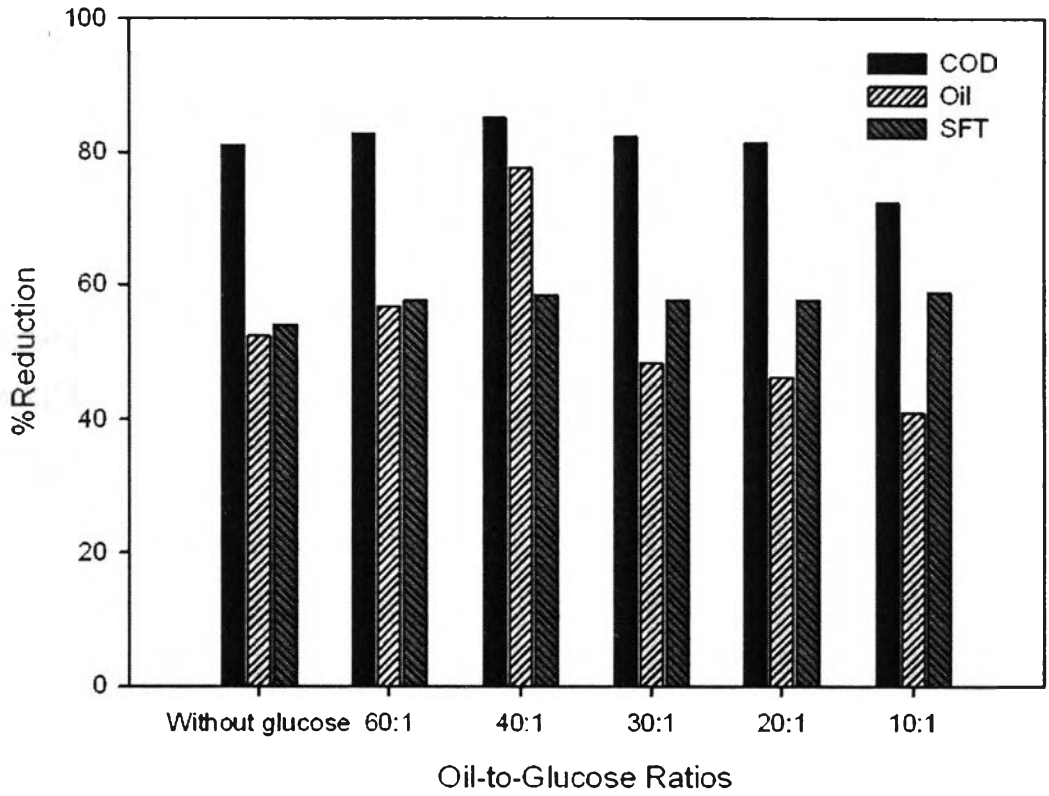

Figure 4.9 The average percentage of COD, oil, and surface tension reduction during steady-state operation at (days 8-14) at different oil-to-glucose ratios.

Figure 4.9 shows comparison of the average percentage of COD, oil, and surface tension reduction during steady-state operation at oil-to-glucose ratio without glucose added, 60/1, 40/1, 30/1, 20/1, and 10/1 oil-to-glucose ratios. It clearly demonstrated that the oil-to-glucose ratio significantly affected the biosurfactant production. At 40/1 oil-to-glucose ratio, the highest COD removal of 85.14% was achieved, suggesting the highest efficiency of the microbe in utilizing palm oil as an organic substance. The percentages of oil and surface tension reductions also ensured that the optimum cycle time for the biosurfactant production by *Pseudomonas aeruginosa* SP4 using SBRs at 40/1 oil-to-glucose ratio.

4.3 Measurement of Biosurfactant Concentration

After the optimization of oil-to-glucose ratio for the biosurfactant production by *Pseudomonas aeruginosa* SP4, a surface tension of culture medium was determined as a function of aeration time in order to investigate the profile of the biosurfactant production. Figure 4.10 shows a surface tension profile at 40/1 oil-to-glucose ratio with an oil loading rate of 2 kg/m^{3}d. It was found that a surface tension of the culture media was slightly decreased during the first 4 h of aeration time but it was rapidly decreased at aeration time of sixth hour before remaining at around 28 to 31 mN/m. The results proved that the biosurfactant should be produced after sixth hour of aeration step then biosurfactant became stable throughout the aeration period, implying that the rate of biosurfactant degradation equals the rate of biosurfactant production. (Cassidy and Huduk, 2001).

Critical micelle dilution (CMD) was performed to examine the biosurfactant concentration. The samples taken during the aeration period of 2d/cycle time were serially diluted, and a surface tension was subsequently measured. Figure 4.11 shows a surface tension of serial dilutions at aeration time of 6, 12, 18, 24, 30, 36, 42, and 48 h. Serial dilution of the sample caused the reduction of biosurfactant concentration. A surface tension significantly increased when the biosurfactant concentration was lower than its CMC. It was found that the dilution of the sample taken from the aeration time of 42 and 48 h from 90:10 to 75:25 of dilution caused an increase in a surface tension from 29 to 31 mN/m to 56.90, and 57.81 mN/m, respectively. Therefore, the CMD of 40/1 oil-to-glucose ratio with 2 d/cycle time at an oil loading rate of 2 kg/m^{3}d was 1.11, representing the biosurfactant concentration of 1.11 times the CMC. When using palm oil as a sole carbon source, the biosurfactant concentration was at 1.05 times the CMC (Pornsunthorntawee *et al.*, 2009). The results exhibited that the glucose that added to the reactor could enhance more biosurfactant concentration when compared to using palm oil as a sole carbon source.

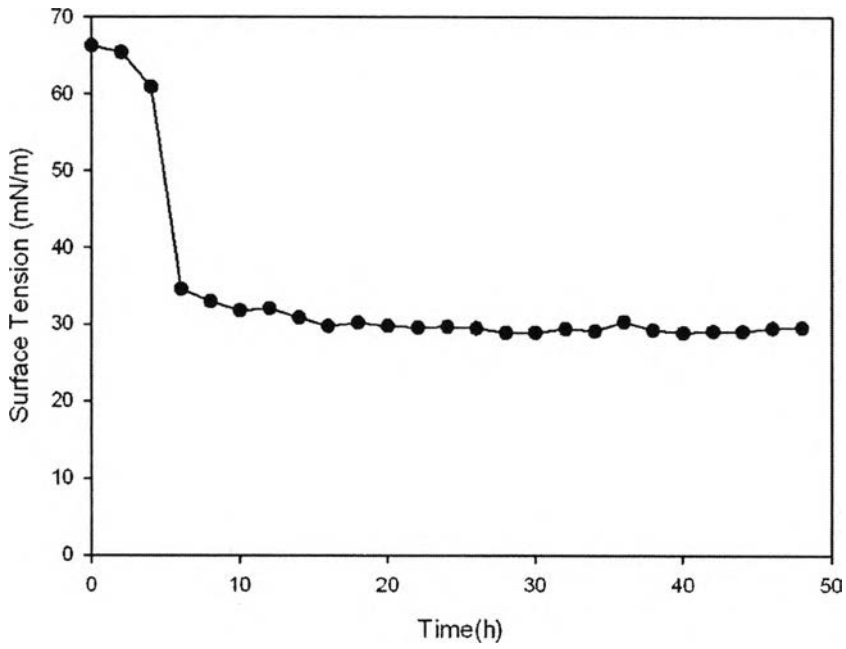

Figure 4.10 A surface tension profile at 40/1 oil-glucose-ratio with an oil loading rate of 2 kg/m^{3}d.

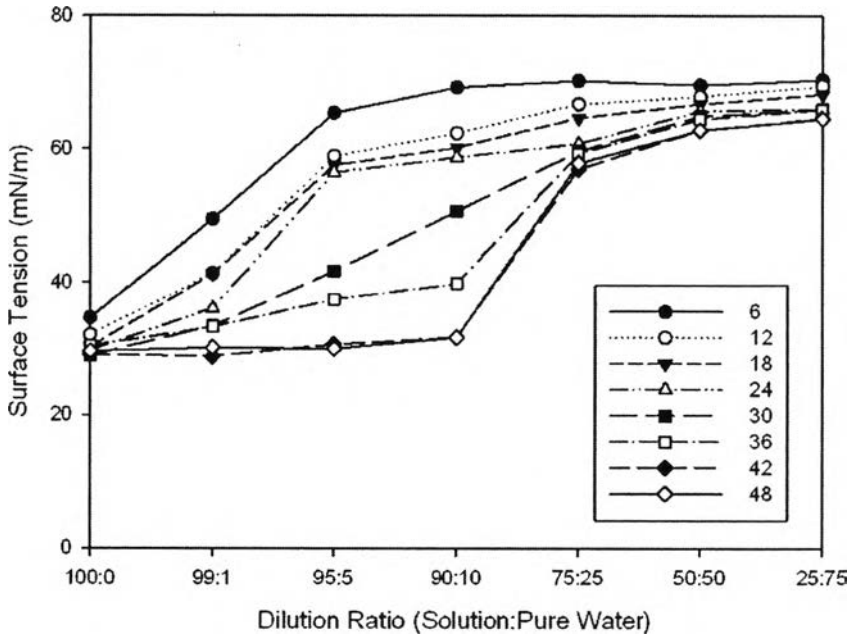

Figure 4.11 A surface tension of serial dilutions at aeration time of 6, 12, 18, 24, 30, 36, 42, and 48 h of 40/1 oil-to-glucose ratio with 2 d/cycle time at an oil loading rate of 2 kg/m^{3}d.